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Newly discovered population of Bumpy Glassfrog, *Centrolene heloderma* (Duellman, 1981), with discussion of threats to population persistence

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Abstract

Since 2011, the Bumpy Glassfrog, *Centrolene heloderma* (Duellman, 1981) was known only from a single location in the Mindo region of Ecuador. We report a newly discovered population located in the area of La Sierra, along Río Alambi, a stream system used heavily for aquaculture of rainbow trout (*Oncorhyncus mykiss*). Threats to population persistence include anthropogenic habitat disturbances such as agriculture and aquaculture, and *Batrachochytrium dendrobatidis* infection. To improve conservation outcomes, we include notes on *C. heloderma* natural history, *Batrachochytrium dendrobatidis* susceptibility, and discuss possible mitigation strategies to protect the species.

Key words

Trout farming; land management; aquaculture; introduced species; Ecuador; *Batrachochytrium dendrobatidis*.

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Introduction

Of the 8 glassfrog species currently known from the Mindo parish (Pichincha province, Ecuador), 2 are categorized as Critically Endangered (*Centrolene heloderma*, *Centrolene ballux*), 1 Endangered (*Centrolene lynchi*), and 2 as Vulnerable (*Nymphargus griffithsi, Centrolene peristictum* (IUCN Redlist 2017). In 2011 a population of *Centrolene heloderma* (Duelman 1981), the bumpy glassfrog, was discovered at Reserva Las Gralarias (00°02′09.6″ S, 078°42′25″ W; Hutter and Guayasamin 2012, Arteaga et al. 2013), though historically the species had been recorded at Quebrada Zapadores and Guajalito (Arteaga et al. 2013; Table 1). Additionally, Hutter and Guayasamin (2012) reported *C. heloderma* from several localities within and adjacent to the Reserva Las Gralarias (RLG): Ballux Creek, Five-Frog Creek, Heloderma Creek, and Hercules Creek. Here we report a newly discovered population of *C. heloderma* in the area of La Sierra, along Río Alambi, a stream system used heavily for aquaculture of rainbow trout (*Oncorhyncus mykiss*). This newly discovered population is geographically separated (ca 6 km) from the known populations in and near RLG by multiple roads and settlements.

Similar to many species in the family Centrolenidae, *C. heloderma* is thought to be threatened by anthropogenic

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Table 1. *Centrolene heloderma* localities (extant and historic).

Locality	Latitude (S)	Longitude (W)
Río Alambi, new population	00°03′34″	078°36′30″
Reserva Las Gralarias, Five Frog Creek	00°01′52″	078°42′22″
Reserva Las Gralarias, Heloderma Creek	00°01′15″	078°42′22″
Reserva Las Gralarias, Hercules Creek	00°01′32″	078°42′15″
14 km W Chiriboga	00°15′55″	078°50′52″
5 km ESE Chiriboga	00°14′43″	078°43′34″
Las Gralarias - Río Santa Rosa	00°02′09″	078°42′25″
8.6 km SE Tandayapa	00°01′59″	078°42′00″
9 km SE Tandayapa	00°01′00″	078°40′59″
13.1 km NW Nono	00°00′09″	078°39′34″

environmental changes, notably habitat disturbances including agriculture and aquaculture (Coloma et al. 2004) and *Batrachochytrium dendrobatidis* (*Bd*) infection (Guayasamin et al. 2014). To protect populations of *C. heloderma* from decline and extirpation, the assessment of potential threats to this species must commence. We present the results of *Bd* testing and suggest that future studies are required to assess potential threats and provide conservation direction at known locales of this species.

Methods

During fieldwork aimed to study the influence of trout farming on glassfrog community composition and the glassfrog skin-associated microbiome (Krynak et al. in preparation), we discovered a new population of *Centrolene heloderma* at Río Alambi. Research permits were issued by the Ministry of the Environment, Ecuador (Ministerio de Ambiente del Ecuador, MAE-DNB-CM-2015-0017).

To assess *Bd* load/prevalence on the skin of *C. heloderma*, skin swabs were collected from 5 of our individuals from the newly documented population and from 4 individuals collected from Reserva Las Gralarias. Swab samples (MW 113, Advantage Bundling) were collected from the dorsum and venter of each animal in a standardized manner, preserved in RNAlater™ solution (Invitrogen), and frozen until sample processing.

Samples were processed at Pisces Molecular as follows. Samples were homogenized (40 seconds at 4500 rpm, 30 s pause, 40 s at 4500 rmp) using a Precellys® Evolution homogenizer. Total DNA was extracted from all samples using the Qiagen/MoBio PowerSoil spin-column DNA purification procedure. Quantitative PCR assay was performed using the following procedure: sample DNA was assayed for the presence of the *Bd* ribosomal RNA Intervening Transcribed Sequence (ITS) region by 45 cycle PCR amplification using a qPCR assay developed at Pisces and an Agilent AriaMx real-time PCR instrument. The reaction master mix contained ROX as passive reference dye for normalizing variations in individual reaction total volumes, and a VIC labeled internal positive control (IPC) (Life Technologies TaqMan Exogenous Internal Positive Control, catalog #4308323) to detect PCR inhibition. The detection sensitivity of this assay is three target sequence molecules (approximately 0.02 zoospore equivalents). Each PCR run included positive and negative controls. Positive DNA controls consisted of DNA prepared from a plasmid constructed at Pisces containing a portion of the *B. dendrobatidis* ribosomal RNA Intervening Transcribed Sequence (ITS) region, and serial ten-fold dilutions of this plasmid DNA from 2.9 x 106 to 2.9 x 100 molecules per reaction were used to generate the standard curve. The negative controls consisted of H_2O in place of template DNA. This reaction remained uncapped during addition of sample DNA to the test reactions, and serves as a control to detect contaminating DNA in the PCR reagents or carryover of positive DNA during reaction set-up. As a comparison to the previous study assessing *Bd* prevalence in *C. heloderma* (Guayasamin et al. 2014), we present *Bd* prevalence as a percentage of *Bd* positive individuals sampled from each population.

Results

New record. Ecuador: Alambi region: Río Alambi (00°03′34″ S, 078°36′30″ W, elev. 2390 m), Katherine L. Krynak observer, 24 April 2017. Audio recordings of calls are deposited at Universidad San Francisco de Quito (LBE-USFQ-2017-001). Number of individuals: 20+.

Identification. We identified *C. heloderma* by its audible calls and morphological features. Several identifiable morphological features facilitate the identification of *C. heloderma*. These include the body size (females SVL 27–32 mm), the brightly cream-colored lower lip, humeral spines in males, the sharply angled rostrum where the lower lips extend far beyond the nostrils, and the pustular dorsal skin texture; these traits distinguish this species from all other glassfrogs of the region; Fig. 1A) (Duellman 1981, Arteaga et al. 2013). Identification by audio recordings and morphological characteristics was confirmed by JMG.

Bd prevalence was found to be 25% (1/4 sampled) at RLG and 0% (0/5 sampled) at this newly discovered population. The single *C. heloderma* to test positive at RLG was found to have a moderate infection load (1.42×10^5 *Bd* target copies present in the sample). Figure 2 shows the distribution of the species, including the new population.

Discussion

Call surveys during the months of March, April, and May 2017 revealed more than 20 male *C. heloderma* calling 5–15 m above the Río Alambi. This may indicate that there was an active breeding population, although neither females nor eggs were observed. Multiple small tributaries, which may also support glassfrog reproduction, flow

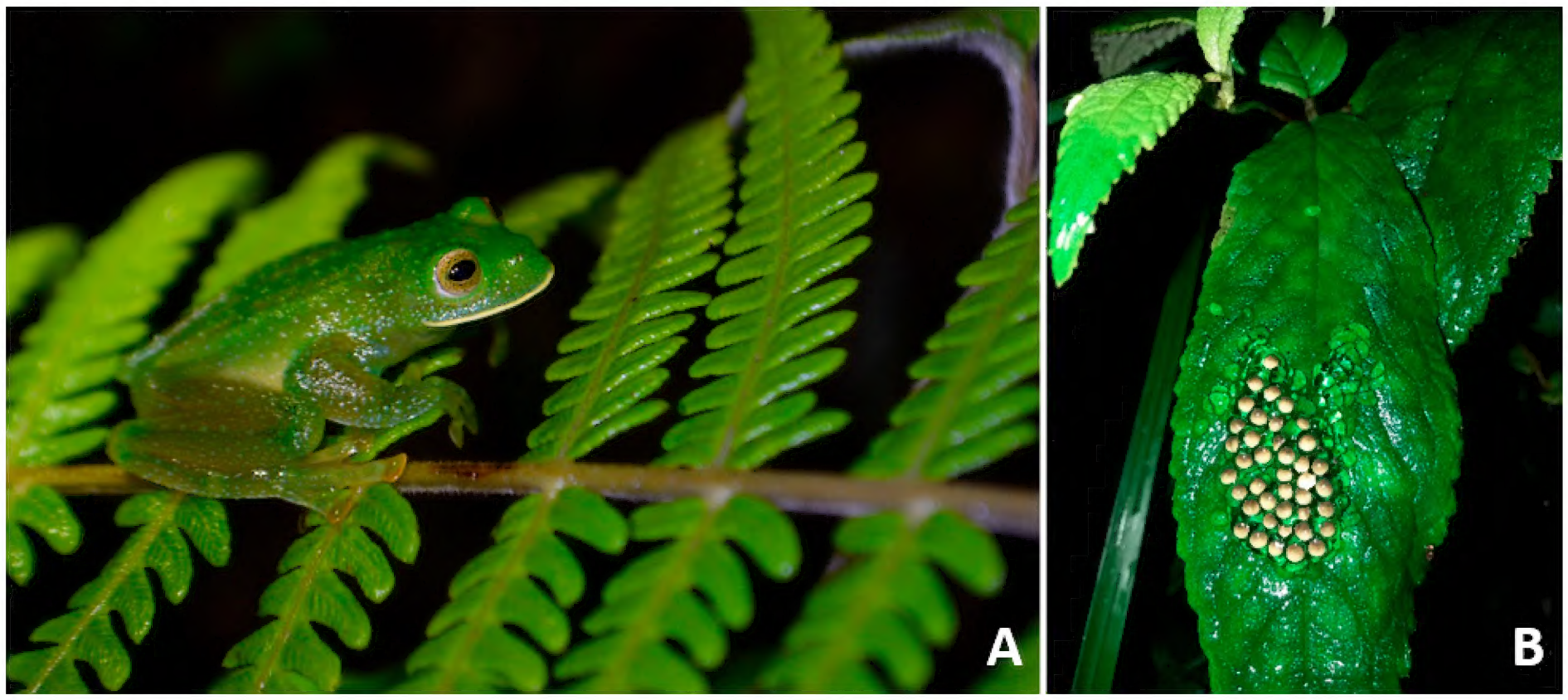

Figure 1. *Centrolene heloderma.* **A.** Adult male; note lip coloration, snout shape, and skin texture. **B.** Eggs on the upper side of a plant leaf.

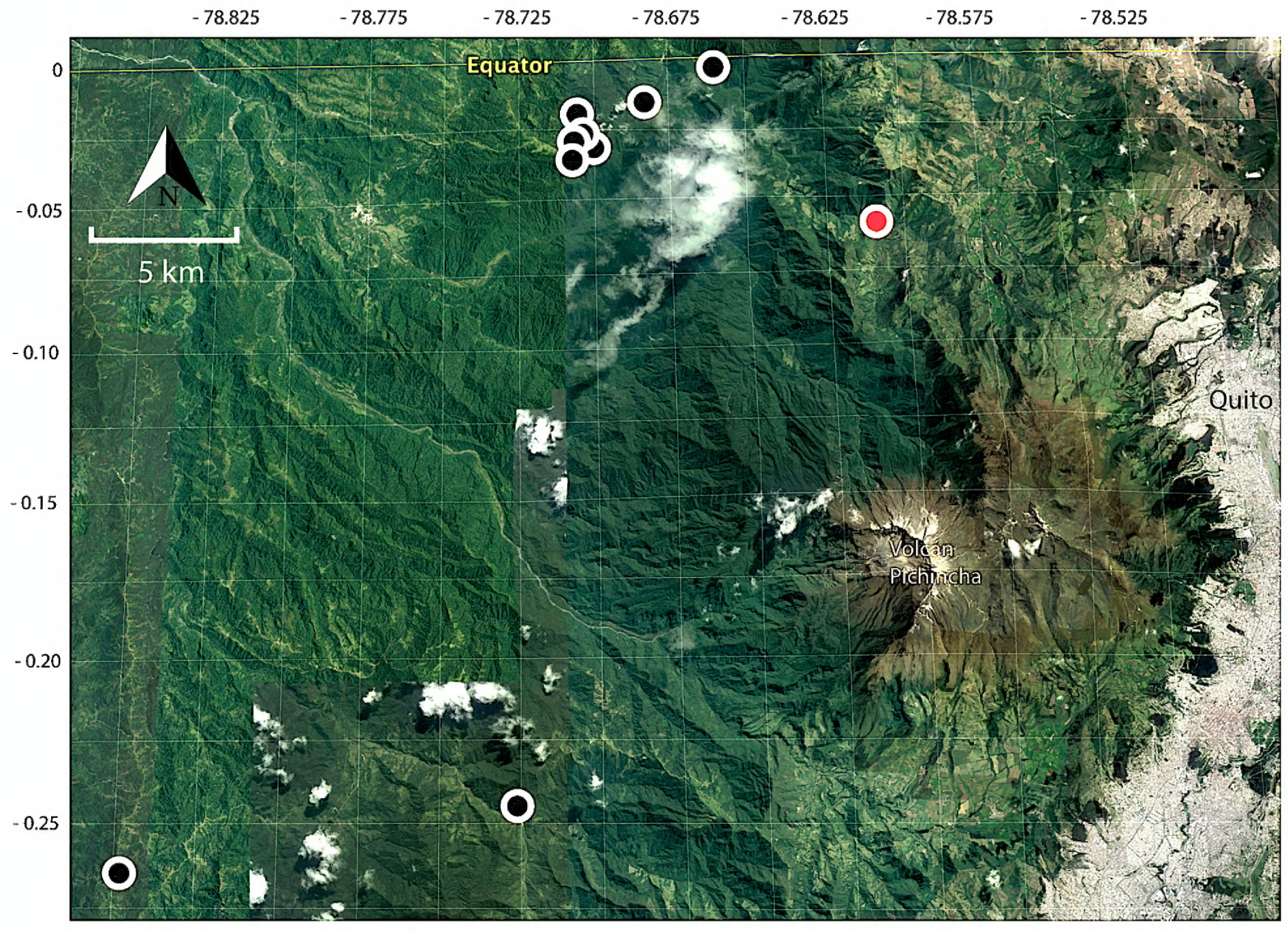

Figure 2. Extant and historic distribution of *Centrolene heloderma* in Ecuador (WGS 84). The newly documented population is in red. Map created by JMG.

into Río Alambi near this site.

Figure 2 shows the distribution of *C. heloderma*. The newly found population is important from a conservation standpoint as *C. heloderma* is Critically Endangered. The finding also indicates that these populations may be resilient to some degree of habitat disruption; at least if disruption is temporally and geographically dispersed, though it is unknown as to whether the population has significantly declined due to such anthropogenic effects. The previously known populations of *C. heloderma* are located along the cobbled road from Quito to Mindo, a road that was initially constructed over 80 years ago according to local residents and that was the only road until the new Quito-coastal highway was finished in 1990. In this area, a buried pipeline carrying heavy-crude oil (Oleoducto de Crudos Pesados; OCP) was constructed

between 2001 and 2003 (documented in the land deeds required for construction; JAL pers. comm.). Aquaculture in the upper Alambi stream system is at least 25 years old according to local land and farm owners. The lower Río Alambi stream system is now extensively used for the aquaculture of Rainbow Trout (*Oncorhyncus mykiss*). Five trout farms in the area extending from Río La Sierra to its confluence with the Río Alambi were included in the surveys of the area. Rainbow Trout and other predatory sport fishes are known to negatively affect amphibian populations by directly consuming amphibian larvae (Gillespie 2001, Pilliod and Peterson 2001, Kats and Ferrer 2003, Bosch et al. 2006, Orizaola and Braña 2006). There might be additional indirect effects of aquaculture on glassfrogs, including reduced water quality and the transmission of diseases such as the freshwater oomycete, *Saprolegnia diclina,* from fish to frog eggs (Martín-Torrijos et al. 2016). Furthermore, the presence of *O. mykiss* might induce behavioral and morphological changes in the larvae of *C. heloderma*, as has been described in another glassfrog, *Nymphargus grandiosonae* (Cochran & Goin, 1970) (Martín-Torrijos et al. 2016).

Active reforestation efforts at RLG are being made and habitat protection is in place to prevent a further decline in the population of *C. heloderma* in the reserve (Bonaccorso 2013, JAL pers. comm.). Two of the streams in which this population is breeding are crossed by the OCP. An access trail and the pipeline route is maintained by OCP workers, who stabilize eroding creek banks where the pipeline crosses. This maintenance includes the removal of vegetation overhanging eroding sites within the 25 m wide zone of legal access. Along all streams where *C. heloderma* occurs, overhanging vegetation is critical to its reproductive success. *Centrolene heloderma* lays up to 29 eggs on top leaves that are overhanging streams (Fig. 1B). Egg deposition sites include plants of the families Araceae, Annonaceae, Euphorbiaceae, Capparaceae, Fabaceae, and Rubiaceae. After hatching, the rheophilic larvae drop into the stream below where they continue to grow and mature (Arteaga et al. 2013). Other maintenance conducted by OCP workers includes the addition of concrete to the stream beds to improve water flow and protect the underlying pipeline. This potentially threatens larvae of *C. heloderma* by removing substrate needed for shelter and increasing their vulnerability to predators (Baber and Babbit 2004). The most obvious threats to *C. heloderma* associated with the OCP include the initial construction and the possibility of pipeline leakage or a catastrophic rupture spilling heavy crude oil into the habitat.

Another potential threat to *C. heloderma* is the fungal pathogen *Batrachochytrium dendrobatidis*. While sample sizes were low at RLG in 2012 and 2013, *Bd* prevalence averaged 17% (1/6 sampled) and 0% (0/1 sampled), respectively (Guayasamin et al. 2014). In 2017, we found the prevalence of *Bd* to be 25% (1/4 sampled) at RLG and 0% (0/5 sampled) in the Río Alambi population. It is unclear as to the mechanism by which *C. heloderma* harbors the *Bd* pathogen in the observed abundances without displaying symptoms (Guayasamin et al. 2014), although the species' skin-associated microbiome is suspected to contribute to this phenomenon and warrants further examination (Belden and Harris 2007). It is possible that anthropogenic habitat changes caused by the OCP pipeline, road construction, and aquaculture may influence susceptibility of *C. heloderma* to *Bd* infection by altering the skin-associated microbiome (Krynak et al. 2015, Krynak et al. 2016).

Information currently available on previously known and newly discovered populations suggest that *C. heloderma* may be able to maintain viable populations in habitats with some anthropogenic modifications, such as secondary forests and partial clearings. However, this species might have very narrow physiological or dispersal constraints, which would explain its small distribution and narrow altitudinal range. Nonetheless, habitat mitigation strategies, including natural and assisted revegetation of stream sides, the minimization of water contamination by the implementation of settling pools, and the prevention of fish escapes may be enough to maintain these populations. This hypothesis requires further study, and the costs and benefits of mitigation (both environmental and human-carried) need to be assessed to prioritize strategies that will improve environmental conditions, protect habitat, and maintain livelihoods of local people.

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Authors' Contributions

KLK, DGW, and SMI collected the data; KLK, JAL, and JMG wrote the text, with significant contributions from all coauthors; KLK provided the audio files, DGW and SMI provided the photographs for figures. JMG produced the map.

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